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Published in:
Molecular Biology of the Cell

DOI:
[10.1091/mbc.5.8.829](https://doi.org/10.1091/mbc.5.8.829)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1994

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Evers, M. E., Titorenko, V. I., Klei, I. J. V. D., Harder, W., & Veenhuis, M. (1994). Assembly of Alcohol Oxidase in Peroxisomes of the Yeast *Hansenula polymorpha* Requires the Cofactor Flavin Adenine Dinucleotide. *Molecular Biology of the Cell*, 5(8), 829-837. <https://doi.org/10.1091/mbc.5.8.829>

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Assembly of Alcohol Oxidase in Peroxisomes of the Yeast *Hansenula polymorpha* Requires the Cofactor Flavin Adenine Dinucleotide

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Submitted November 19, 1993; Accepted June 17, 1994
Monitoring Editor: Randy W. Schekman

The peroxisomal flavoprotein alcohol oxidase (AO) is an octamer (600 kDa) consisting of eight identical subunits, each of which contains one flavin adenine dinucleotide molecule as a cofactor. Studies on a riboflavin (Rf) auxotrophic mutant of the yeast *Hansenula polymorpha* revealed that limitation of the cofactor led to drastic effects on AO import and assembly as well as peroxisome proliferation. Compared to wild-type control cells Rf-limitation led to 1) reduced levels of AO protein, 2) reduced levels of correctly assembled and activated AO inside peroxisomes, 3) a partial inhibition of peroxisomal protein import, leading to the accumulation of precursors of matrix proteins in the cytosol, and 4) a significant increase in peroxisome number. We argue that the inhibition of import may result from the saturation of a peroxisomal molecular chaperone under conditions that normal assembly of a major matrix protein inside the target organelle is prevented.

INTRODUCTION

The yeast *Hansenula polymorpha* is able to utilize methanol as the sole source of carbon and energy (Veenhuis *et al.*, 1978). Growth on this compound is associated with the induction of a specific set of proteins, which catalyzes the conversion of methanol into biomass and energy concomitant with the development of peroxisomes (Veenhuis *et al.*, 1979). The initial step of methanol metabolism in *H. polymorpha* is mediated by a peroxisomal enzyme, alcohol oxidase (AO)¹, that catalyzes the oxidation of methanol into formaldehyde and H₂O₂, using oxygen as the electron acceptor (van Dijken *et al.*, 1976). The active form of AO is an octamer of ~600 kDa, consisting of eight identical subunits each of which contains a flavin adenine dinucleotide (FAD) molecule as the cofactor (Kato *et al.*, 1976). In vivo the activity of AO is confined to the peroxisomal matrix (Veenhuis *et al.*, 1976). However, the molecular mechanisms in-

involved in targeting and translocation of cytosolic AO precursors into the peroxisomal matrix and the subsequent assembly into the functional enzyme have not yet been elucidated. Only recently the peroxisomal targeting signal of AO has been identified and was shown to consist of a C-terminal tripeptide (-ARF) (Hansen *et al.*, 1992).

AO octamerization most probably is not a spontaneous process but instead dependent on specific factors (chaperones) present only inside peroxisomes (Roa and Blobel, 1983; Distel *et al.*, 1987; van der Klei *et al.*, 1989). On the other hand, active oligomeric AO is present in the cytosol of peroxisome-deficient mutants of *H. polymorpha* (van der Klei *et al.*, 1991a). This indicates that the putative factors involved in AO assembly apparently do not require the specific acidic environment of the target organelle (Nicolay *et al.*, 1987) for proper functioning.

Apart from specific molecular chaperones, also the cofactor FAD may be required for AO oligomerization. The role of FAD-binding in AO assembly has been studied before using mutated AO genes that contain directed mutations in the FAD-binding fold. Unfortu-

¹ Abbreviations used: AO, alcohol oxidase; FAD, flavin adenine dinucleotide; Rf, riboflavin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

nately, these mutations also strongly affected the import competence of the synthesized protein and thus severely hampered a proper analysis of the effect of the mutation on AO assembly/activation (de Hoop *et al.*, 1991).

For this reason we have chosen another approach and studied AO assembly in a riboflavin (Rf) auxotrophic mutant of *H. polymorpha*. The use of this mutant allowed us to manipulate the amount of Rf, the precursor of FAD, available for the cells. However, the structural gene of AO is unaltered and hence the properties of the AO protein with respect to targeting and translocation. The results of these studies are described in this paper.

MATERIALS AND METHODS

Organisms and Growth Conditions

Hansenula polymorpha NCYC 495 (*leu*⁻) and mutant *rif1*, derived from this strain, were used in all experiments. Cells were grown in batch cultures at 37°C in mineral medium using 0.5% (wt/vol) glucose, 0.5% (vol/vol) glycerol, 0.5% (vol/vol) methanol or a mixture of 0.5% (vol/vol) glycerol and 0.5% (vol/vol) methanol as carbon sources (van Dijken *et al.*, 1976). The mutant *rif1* was grown in the presence of different concentrations of Rf, ranging from 0 to 1 mM. Before the shift to glycerol + methanol or methanol-containing media, cells were extensively precultured on glucose in the presence of 0.6 mM Rf. Growth was monitored by measuring the optical density at 663 nm in a Vitatron colorimeter (Vital Scientific, Dieren, The Netherlands).

Mutant Isolation

Cells of *H. polymorpha* NCYC 495 (*leu*⁻) were mutagenized using N-methyl-N'-nitro-nitrosoguanidine (250 µg/ml, 10 min) and subsequently incubated for 24 h in mineral medium containing 0.5% (vol/vol) methanol as the sole carbon source. After Nystatin enrichment (25 U/ml, 15 min) of Rf auxotrophs, cells were spread on agar plates containing 0.5% (vol/vol) methanol and 0.5 mM Rf (Cregg *et al.*, 1990). Resulting colonies were replica plated on methanol plates lacking Rf and checked for growth. One Rf-auxotrophic strain was selected, backcrossed three times with the parental strain by the method described before (Waterham *et al.*, 1992a), and used in further studies.

Biochemical Methods

Crude extracts were prepared as described previously (van der Klei *et al.*, 1991a). AO activity was measured according to Verduyn *et al.* (1984); in some cases 1 mM FAD was added to the crude extract and incubated for 1 h at room temperature before enzyme measurements. Octameric AO protein was partly purified by sucrose density centrifugation (Bellion and Goodman, 1987). Sucrose gradients were harvested by taking 0.45-ml samples from the top. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (BSA) as standard. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970), and Western blotting was performed as described by Khyse-Andersen (1984). FAD concentrations were determined by measuring emission at 521 nm, using a luminescence spectrometer (Perkin Elmer LS50B, Norwalk, CT) and standards of known concentrations of FAD.

Cell Fractionation

Protoplasts were prepared by treatment of whole cells with Zymolyase 20-T (ICN Biomedicals, Costa Mesa, CA). For fractionation

studies, protoplasts were homogenized and subjected to differential centrifugation as described previously (Douma *et al.*, 1985).

Electron Microscopy

For studies of the cell morphology, whole cells were fixed in 1.5% (wt/vol) KMnO₄ at room temperature for 20 min. Spheroplasts were fixed in 6% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 at 0°C for 60 min. Postfixation was performed in a mixture of 1% (wt/vol) OsO₄ and 2.5% (wt/vol) K₂Cr₂O₇ in the same cacodylate buffer at room temperature for 60 min.

After fixation the samples were poststained in 1% (wt/vol) uranyl acetate in distilled water for 8–16 h, dehydrated in a graded ethanol series, and embedded in Epon 812 (Serva, Heidelberg, Germany). Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 (Mahwah, NJ). The average number of peroxisomes in thin sections of cells was established by random counting as described before (Veenhuis *et al.*, 1978).

(Immuno)cytochemistry

Cytochemical localization of AO activity was performed by the cerium-based method (Veenhuis *et al.*, 1976). Immunocytochemistry was performed on ultrathin sections of Lowicryl K₄M-embedded cells by the protein A/gold method (Slot and Geuze, 1984), using polyclonal antibodies raised against AO, catalase, and dihydroxyacetone synthase.

RESULTS

Isolation of Mutants and Growth

Mutagenesis of wild-type *H. polymorpha* and subsequent Nystatin enrichment resulted in the isolation of 27 strains, which were dependent on Rf for growth on solid agar plates. One of these mutants, harbouring a monogenic recessive mutation, was selected, backcrossed three times with the parental strain, and used for further analysis. This mutant, designated *rif1*, was strictly dependent on Rf for growth in mineral media, containing either glucose, glycerol, glycerol + methanol, or methanol as carbon sources. In the absence of Rf, growth was impaired and could also not be restored by either flavin mononucleotide or FAD. Growth experiments indicated that on glucose full restoration of both growth rate and yield of mutant *rif1* was obtained in the presence of 0.25 mM Rf; identical results were obtained on glycerol or glycerol/methanol mixtures although under these conditions enhanced Rf concentrations, namely 0.5 mM, were required. However, on 0.5% methanol alone growth remained affected even in the presence of 1 mM Rf (Table 1, columns 2 and 3). Most likely, this has to be explained by the reduced specific AO activities, observed under these conditions (Table 1, column 5) (for details see below).

Ultrastructural Analysis

Detailed analyses of KMnO₄-fixed cells of variously grown *rif1* mutant cells revealed that Rf-limitation distinctly affected the patterns of peroxisome proliferation in the cells. In fact two remarkable alterations were observed. These included that in cells grown in batch cul-

Table 1. Growth and specific AO activity in an Rf auxotrophic mutant (*rif1*) or *H. polymorpha*

Strain	[Rf] ^a	t _d ^b	OD ₆₆₃ ^c	AO protein levels ^d	AO activity ^e
<i>rif1</i>	0	—	0.4	60%	0.30
	0.2	>40	1.8	60%	0.54
	0.4	33	2.3	70%	0.52
	0.6	29	3.3	150%	1.62
	0.8	14	3.5	110%	1.92
	1.0	14	3.8	n.d.	1.63
Wild-type	0.6	4	3.3	100%	2.56

Cells were grown in batch cultures in the presence of various amounts of Rf. —, no growth; n.d., not determined.

^a Concentrations (mM) of Rf in the cultivation media.

^b Doubling times (h) during exponential growth on methanol-containing media.

^c Final optical density, measured at 663 nm, of cell cultures after 70 h of incubation on methanol-containing media.

^d Relative amount of AO protein in crude extracts, prepared from cells incubated for 24 h in methanol-containing media. AO protein levels are determined by laser-densitometric scanning of Western blots, decorated with specific α -AO antibodies; identical amounts of proteins were loaded on a gel (amount of AO protein in wild-type cells is set to 100%).

^e Specific AO activity in crude extracts, prepared from cells incubated for 24 h in methanol-containing media. AO activity is expressed as μ mole methanol converted \cdot min⁻¹ \cdot mg protein⁻¹ (U/mg protein).

tures on either methanol or glycerol/methanol mixtures at decreasing concentrations of Rf (ranging from 1.0—0.2 mM) the size of the individual peroxisomes was reduced, whereas the number of organelles per cell had considerably increased. Representative examples of this are shown in Figure 1 and 2. As is clear from these pictures, cells grown at 1.0 mM Rf contain peroxisomes comparable to those in wild-type cells (size up to \sim 1 μ m), whereas at 0.2 mM Rf the average size of the or-

ganelles amounted 0.3 μ m. However, the mean number of organelles per cell had increased from 2.3 (at 1.0 mM Rf) to 5.9 (at 0.2 mM Rf).

Sections of glutaraldehyde/OsO₄-fixed spheroplasts revealed that in methanol- or glycerol/methanol-grown cells the individual peroxisomes contained crystalline inclusions. Cytosolic crystalloids or protein aggregates (as for instance evident in peroxisome-deficient mutants of *H. polymorpha*) (Veenhuis *et al.*, 1992) were never observed. However, in Rf-limited cells the peroxisomal matrix contained, besides AO crystalloids, also small proteinaceous aggregates (Figure 1B and 3). Immunocytochemical evidence was obtained that these aggregates contained AO protein.

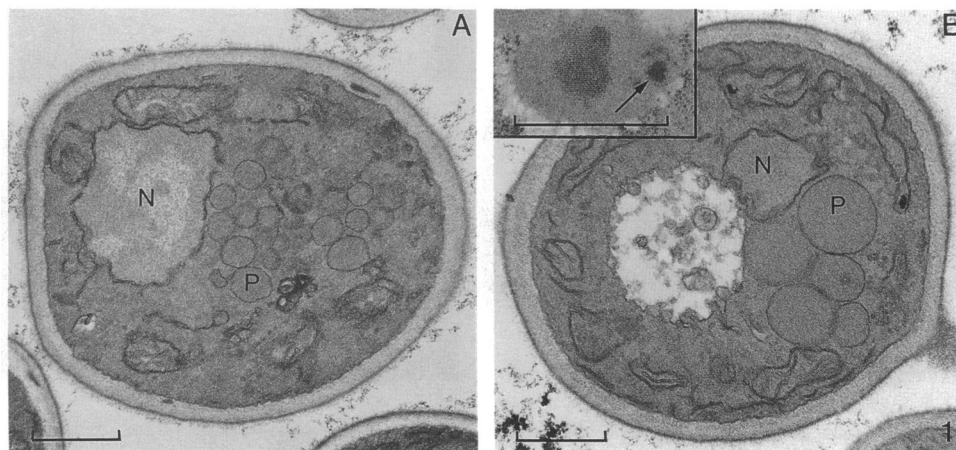
Cytochemistry and Immunocytochemistry

After cytochemical staining experiments for the localization of AO activity, specific reaction products were invariably confined to the peroxisomal matrix, both in wild-type and in *rif1* mutant cells, independent of the Rf-concentration in the growth media (Figure 4). However, in the *rif1* mutant AO protein was also present in the cytosol and in the nucleus, as was evident after immunocytochemical experiments using specific antibodies against AO and protein A/gold. This indicates that import of inactive AO precursors into peroxisomes is affected during Rf limitation. The intensity of the cytosolic labeling was increased on profiles of cells, grown at decreasing concentrations of Rf (Figure 5). Comparable labeling patterns were observed for the second major peroxisomal enzyme dihydroxy-acetone synthase (Figure 4) and catalase. These enzymes do not contain FAD but nevertheless were only partially imported in the *rif1* mutants, grown under Rf-limiting conditions.

Synthesis and Assembly of AO Protein

To determine whether Rf-limitation affected the intracellular levels of AO protein in the *rif1* mutant, cells

Figure 1. Overall morphology of *rif1* cells grown for 24 h on media containing a mixture of glycerol and methanol supplemented with 0.2 mM Rf (A) or 1.0 mM Rf (B) (KMnO₄). In *rif1* cells grown at 0.2 mM Rf, a relatively large number of small peroxisomes is present compared to the organelles present in cells grown at 1.0 mM Rf. The peroxisomal matrix contains, besides AO, crystalloids also protein aggregates (inset Figure 1B, glutaraldehyde, OsO₄). N, nucleus; P, peroxisome. Bar, 0.5 μ m.



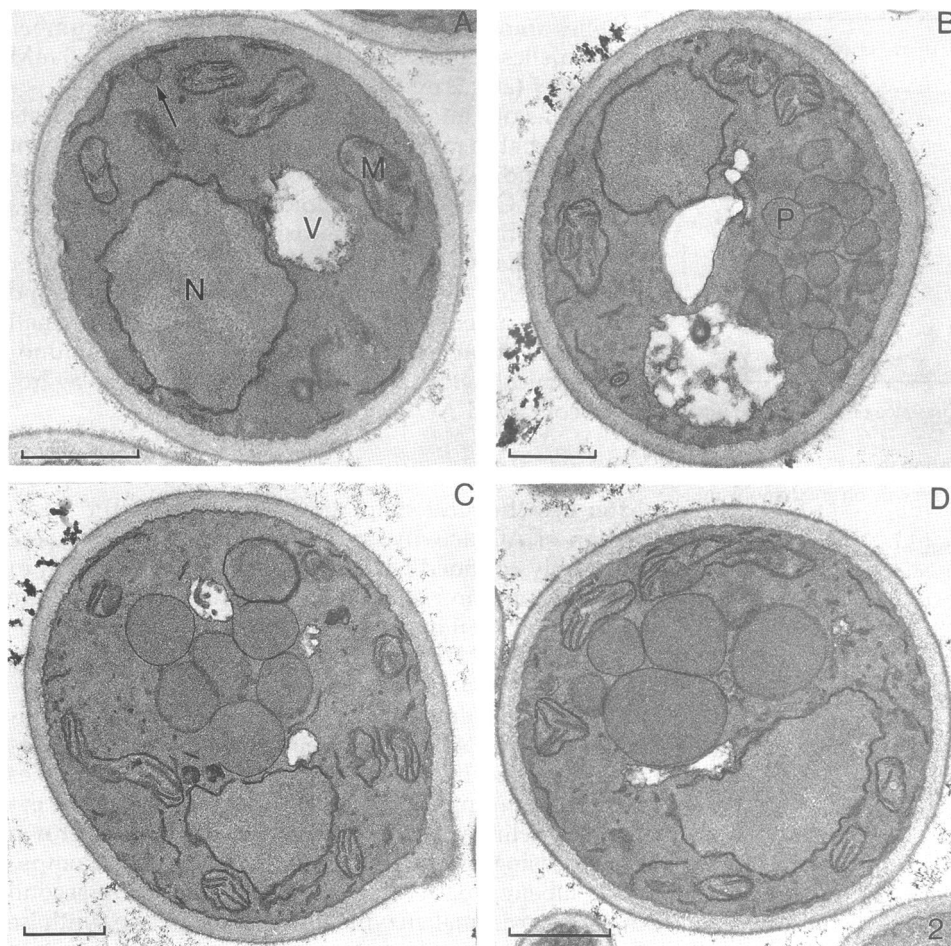


Figure 2. Overall morphology of glucose-grown inoculum cells (A) and after incubation for 24 h in methanol-containing media in the presence of 0.2 mM Rf (B) and 0.8 mM Rf (C). (D) Wild-type control cells are shown. As normal wild-type cells (Veenhuis *et al.*, 1979), glucose-grown *rif1* cells generally contain a single small peroxisome (arrow). Many small peroxisomes develop during growth of cells at 0.2 mM Rf (B); lower numbers of increased size are present at enhanced Rf concentrations (C) and in wild-type controls (KMnO_4). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar, 0.5 μm .

were grown in methanol-containing media supplemented with various concentrations of Rf. The results of Western blotting experiments, an example of which is shown in Figure 6, indicated that the amount of AO protein in crude extracts from *rif1* cells grown in the presence of 0.6 mM Rf could even exceed those of wild-type control cells. Thus, the amounts of AO protein in *rif1* cells were correlated to the concentration of Rf in

the growth medium (Table 1, column 4). Enzyme measurements revealed that also the specific AO activity varied with the Rf concentrations in the growth medium. As shown in Table 1, an increase in the AO enzyme activity was detected concomitant with increasing Rf concentrations in the growth media. However, the specific AO activity in crude extracts of *rif1* cells never exceeded that of wild-type cells, this in contrast to the

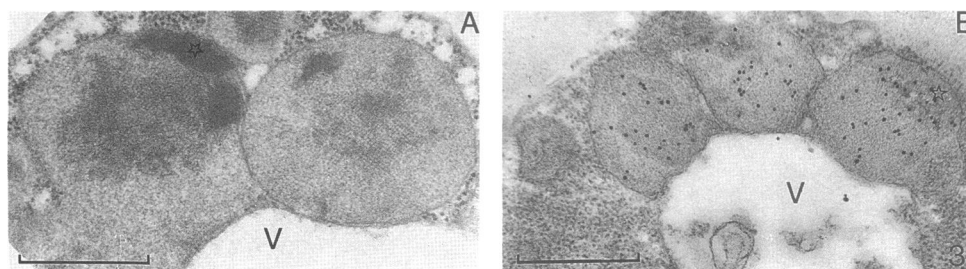
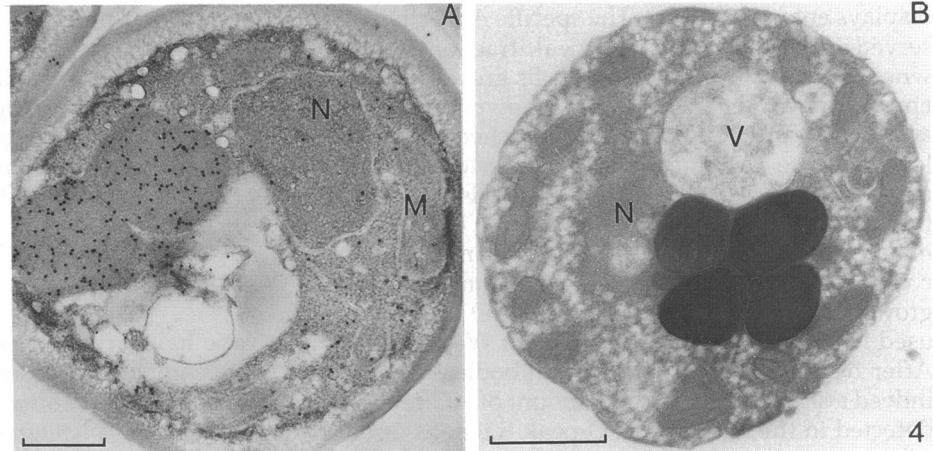


Figure 3. (A) Detail of a cell grown on methanol in the presence of 0.6 mM Rf. In the individual peroxisomes, crystalloids and electron dense aggregates can be observed (*). (B) Results of immunocytochemical experiments using specific antibodies against AO and protein A/gold. Labeling is located on the peroxisomal matrix, both on the crystalloids and the peroxisomal aggregates (glutaraldehyde, OsO_4). For immunocytochemistry sections are bleached before labeling. V, vacuole. Bar, 0.5 μm .

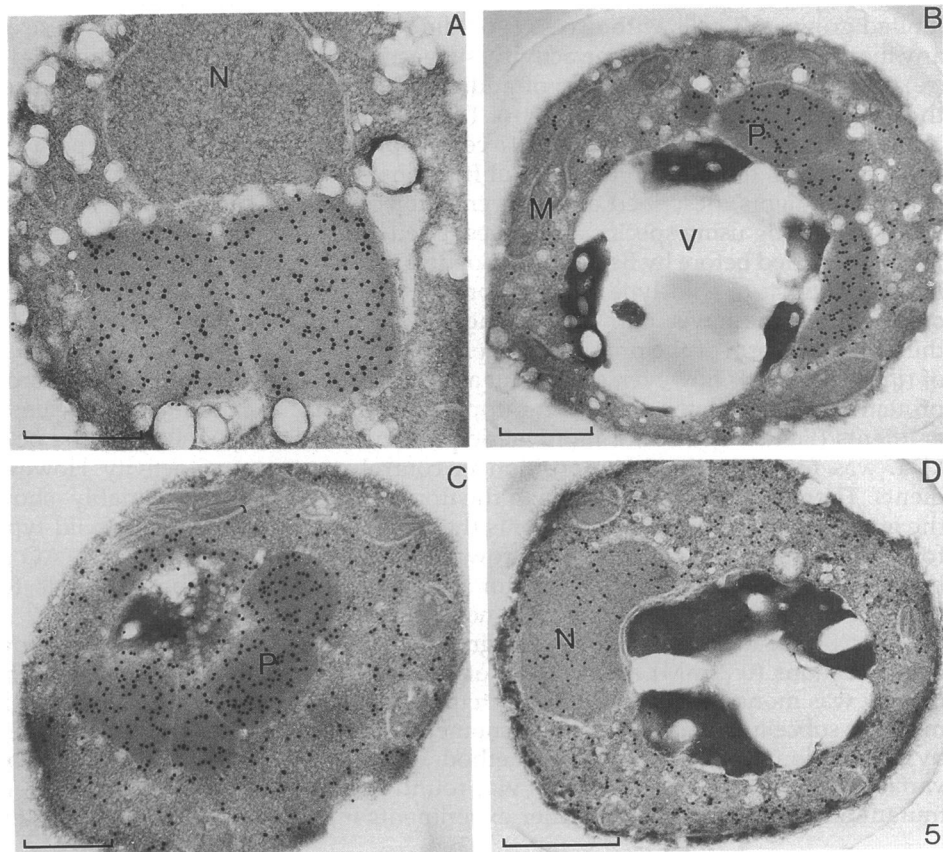
Figure 4. Immunocytochemical demonstration of dihydroxyacetone synthase in both peroxisomes and the cytosol of mutant cells, grown on methanol in the presence of 0.6 mM Rf (A) (glutaraldehyde, Lowicryl; anti-dihydroxyacetone synthase/protein A/gold). (B) Subcellular location of AO activity in peroxisomes of these cells after incubations of spheroplasts with CeCl_3 and methanol (glutaraldehyde, OsO_4). M, mitochondrion; N, nucleus; V, vacuole. Bar, 0.5 μm .



AO protein levels (see above). These results therefore lead to the conclusion that in *rif1* cells AO protein was not efficiently assembled/activated. To study this aspect in more detail, we determined the specific activity and FAD contents of the active AO enzyme. For this purpose crude extracts were subjected to sucrose gradient centrifugation, which resulted in the separation of octameric

AO from other oligomeric forms (Bellion and Goodman, 1987). In all cases, both for wild-type and *rif1* extracts, AO activity was only detectable in the lower part of the gradients (at ~15% sucrose); specific AO peak activities coincided with the peak fractions of purified octameric AO used in a control gradient. These results confirm earlier observations that invariably only octameric AO

Figure 5. Immunocytochemical experiments, using α -AO antibodies and protein A/gold on ultrathin sections of wild-type cells (A) and mutant cells grown in the presence of 1.0 mM Rf (B), 0.6 mM Rf (C), and 0.2 mM Rf (D), respectively. In the wild-type control cells labeling is confined to the peroxisomal matrix; in the variously grown mutant cells however also cytosolic labeling, including in the nucleus (D), is evident. The cytosolic labeling intensities increase with decreasing mounts of Rf supplemented to the cultivation media (glutaraldehyde, Lowicryl; uranyl acetate). M, mitochondrion; N, nucleus; V, vacuole. Bar, 0.5 μm .



displays enzyme activity. The specific AO activities, observed in the octameric AO peak fractions, increased when extracts were used from cells grown in the presence of increasing amounts of Rf; however, specific AO activities in these samples never reached the wild-type levels (Figure 7A). These results suggested that the specific activity of octameric AO in *rif1* cells was related to the amount of Rf available for growth of the cells. To obtain additional evidence for this, samples from the combined octameric AO peak fractions of variously grown cells displaying equal total AO activities were used for SDS-PAGE and subsequent Western blotting. After decoration with specific antibodies against AO, indeed a significantly higher amount of AO protein was detected in the octameric AO peak fractions of the *rif1* cells grown under Rf-limitation. As indicated in Figure 7A, the specific activity of the AO octamers in *rif1* cells may decrease until 40% of the activity of wild-type octamers. FAD measurements revealed that the FAD/AO protein ratios in the octameric-AO-containing fractions of the *rif1* cells had also significantly decreased, compared to wild-type cells. The FAD/AO octamer ratio of AO from *rif1* cells ranged from 50 to 70% of that observed in wild-type cells (Figure 7B). Summarizing, Rf limitation strongly affected both the specific enzyme activity of octameric AO in *rif1* cells and the total amount of FAD bound to AO octamers.

Preincubations of crude extracts prepared from Rf-limited grown *rif1* cells with excess FAD did not cause any increase in the AO enzyme activity, suggesting that (re)activation because of binding of additional FAD to the AO octamers did not occur *in vitro*. To test whether all AO protein synthesized in the *rif1* cells is properly assembled into the octameric form, all fractions of the sucrose gradients, described above, were subjected to Western blotting using specific antibodies raised against AO. As reported before by Bellion and Goodman (1987), in crude extracts of wild-type cells, AO protein was only detectable in the lower part of the gradient in which the octameric form of the protein sediments. In the case of *rif1* mutant cells however, significant amounts of AO protein were also detectable in the upper part of the gradients (Figure 8). BSA, a monomeric protein of 68 kDa, was found in the same position in control gradients. Therefore, the AO detected in the upper part of the gradients most probably represents the monomeric form. In crude extracts of *rif1* cells grown at relatively high concentrations of Rf only a minor amount of the total AO protein was present in the monomeric form (1% at 1.0 mM Rf). However, in cells grown at low Rf concentrations (0.2 mM) almost 30% of the total AO protein was monomeric (Figure 7B). Attempts to determine the subcellular location of the monomeric AO pool by conventional cell fractionation methods failed; invariably only octameric AO protein was found in both mutant and wild-type cells. Further experiments indi-

cated that this was because of proteolytic degradation of monomeric AO during the rather time-consuming fractionation procedure. However taken together with the (immuno)cytochemical results, our data strongly suggest that the cytosolic pool of AO (which shows no enzyme activity; compare (Figure 4B) is monomeric, the octameric, active AO being confined to the peroxisomal matrix.

DISCUSSION

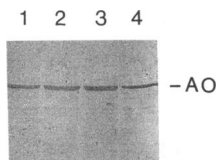
We have investigated the role of the cofactor in the *in vivo* import and assembly of an organellar flavo-protein, namely peroxisomal AO, in a Rf auxotrophic mutant (*rif1*) of *H. polymorpha*. These studies indicated that the availability of Rf, and thus FAD, is of major importance in this respect. Rf limitation not only leads to defects in AO translocation and assembly, it also interferes with the proliferation of peroxisomes.

A serious concern with respect to our data involved the specificity of the effect of Rf limitation, because the reduction in growth on methanol could for instance also be explained as a general effect of FAD depletion in the cells. However, the latter possibility is clearly ruled out by the results obtained with cells grown on glycerol/methanol mixtures. Under these conditions methanol principally acts as an inducer for full derepression of the AO gene (Hansen *et al.*, 1992). Because growth was not affected in these cultures, the cells did not experience a general energy and/or FAD limitation at the lowered Rf concentrations used. However, the overall response of the cells to Rf limitation with respect to AO synthesis/assembly and peroxisome proliferation was fully identical in both methanol- and glycerol/methanol-grown cells and thus leave little doubt that the effects observed in cultures on methanol alone were indeed specific and did not reflect a secondary effect because of a general FAD depletion of the cells.

The cytochemical experiments revealed that, irrespective of the extent of Rf-limitation, AO activity was confined to the peroxisomal matrix. The biochemical experiments revealed that, as in wild-type cells, also in *rif1* cells only the octameric form of AO displayed enzyme activity. However, octameric AO protein from *rif1* cells invariably showed a reduced specific activity, compared to wild-type AO. Under these conditions also a reduced FAD/AO protein ratio was observed, which probably accounts for the observed decrease in AO specific activity.

In addition, Rf-limitation also led to defects in AO translocation/assembly in *rif1* cells. Our data furthermore suggested that in the absence of FAD oligomerization of AO inside peroxisomes is prevented. This raises the question on the minimum amount of FAD that is required to facilitate AO octamerization. In our experiments a minimal FAD/AO protein ratio of 3.6

Figure 6. Western blots, using α -AO antibodies, prepared from crude extracts of wild-type *H. polymorpha* and mutant *rif1*, showing expression levels of AO protein in relation to the amounts of Rf present in the cultivation media. Cells were grown in 0.5% methanol-containing media in the presence of different concentrations of Rf and harvested after 24 h of incubation. Equal amounts of protein were loaded in each lane. Lane 1: wild-type, 0.6 mM Rf. Lane 2: *rif1*, 0.8 mM Rf. Lane 3: *rif1*, 0.6 mM Rf. Lane 4: *rif1*, 0.2 mM Rf.



was observed. These data are in line with the values reported by Giuseppin *et al.* (1988) for octameric AO in methanol-limited wild-type *H. polymorpha*. Taking into account the possible deviation as a result of the applied biochemical procedures, these results suggest that at least four FAD molecules should be available to facilitate proper AO octamerization. It can of course not be ruled out that the observed value of 3.6 indeed is an average value between two extremes and consequently, that AO octamers may exist containing less than four FAD molecules. On the other hand, as suggested before, binding of FAD and AO import are independent processes (van der Klei *et al.*, 1989, 1991a). Therefore, redistribution of FAD from already formed AO octamers may play a role in the final amount of FAD bound to each AO molecule. Thus, FAD previously bound to octameric AO may partly dissociate from the mature protein and be used for the assembly of newly imported AO protein.

However, octameric AO apparently is not able to rebind FAD *in vitro*. This is indicated by our findings that addition of FAD to crude extracts of Rf-limited mutant cells did not lead to an increase in specific AO activity. These results are in line with previous experiments that showed that also *in vivo* FAD does not reassociate with AO octamers, from which FAD was previously released by potassium cyanide (KCN) treatment of the cells (van der Klei *et al.* 1991b). A second key question is why FAD limitation leads to import defects. Our results showed that this is not only true for AO but also for other major matrix proteins, namely catalase and dihydroxyacetone synthase; therefore, FAD limitation may lead to a general peroxisomal import defect in *H. polymorpha*. Our explanation for this phenomenon is based on the observation that the peroxisomal matrix contains small proteinaceous aggregates in Rf-limited mutant cells. These aggregates most probably represent incorrectly assembled matrix proteins, including AO, as was evident from immunocytochemistry. Based on these results the following sequence of events may be proposed. The primary effect of FAD limitation apparently includes the prevention of correct folding and assembly (octamerization) of monomeric AO, which was already imported into peroxisomes. Accumulation of nonassembled AO could result in the saturation of putative

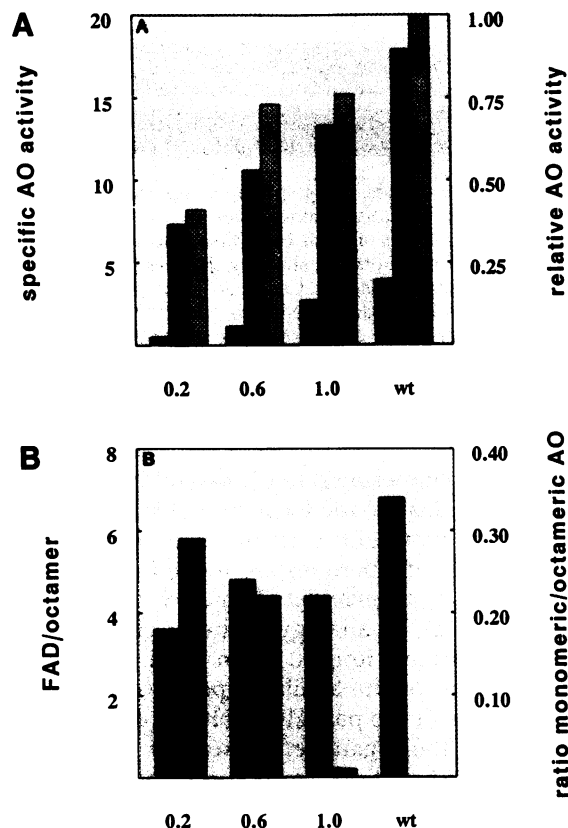


Figure 7. AO assembly and activation in a Rf auxotrophic mutant (*rif1*) of *H. polymorpha*. Cells were grown for 24 h in batch cultures on methanol in the presence of various amounts of Rf (0.2, 0.6, and 1.0 mM Rf, respectively). As a control, wild-type cells were grown in the presence of 0.6 mM Rf. (A) The specific activity of AO in crude extracts (■ U/mg protein) increased with increasing amounts of Rf added but never reached that of wild-type cells. Similarly, the specific activity (and thus the relative activity) of AO octamers is related to the amount of Rf added to the medium. The specific activity of AO octamers (■ U/mg octameric AO protein) was determined using octameric AO peak fractions of sucrose gradients. The AO contents of these fractions was estimated by densitometric scanning of Coomassie Brilliant Blue-stained SDS-PAGE protein gels of these fractions. The relative activity of AO octamers (■) (compared to that of AO from wild-type cells, which was set to 1) was determined as follows: samples from octameric peak fraction containing equal AO activity were subjected to SDS-PAGE and used for Western blotting. The blots were decorated with specific α -AO antibodies. The relative amounts of AO protein were subsequently determined by densitometric scanning. (B) Number of FAD molecules bound per AO octamer (■) and ratios of monomeric/octameric AO (■). The number of FAD molecules/AO octamer was calculated from the amounts of AO protein present in the octamer-containing fractions; FAD levels were determined in the supernatants of trichloroacetic acid precipitates of these fractions. The monomeric/octameric AO ratios were determined as follows: crude extracts were subjected to sucrose gradient centrifugation. Subsequently, equal volumes of the respective monomeric and octameric AO peak fractions (compare Figure 8) were electrophoresed and the blots decorated with specific α -AO antibodies. Data are expressed as the ratio of the amounts of monomeric AO and octameric AO, determined after laser-densitometric scanning of the blots.

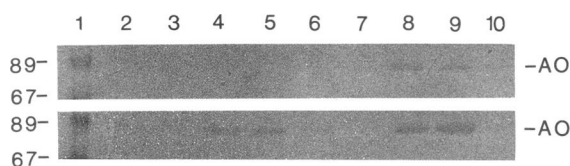


Figure 8. Western blots, using specific α -AO antibodies, prepared from the various fractions obtained after sucrose gradient centrifugation of equal amounts of crude extracts of wild-type *H. polymorpha* (top) and mutant *rif1* (bottom), showing the presence of monomeric AO protein in mutant cells only. Cells were grown in 0.5% methanol-containing media in the presence of 0.6 mM Rf and harvested after 24 h of incubation. Lane 2 represents the top fraction. Lane 10 represents the bottom fraction of the gradient. Lane 1, Molecular weight markers; lanes 4 and 5, monomeric AO; lanes 8 and 9, octameric AO.

peroxisomal chaperones (e.g., assembly factors) and subsequently, lead to the formation of intraperoxisomal aggregates. At present, evidence is accumulating that such molecular chaperones are indeed present in peroxisomes of *H. polymorpha* (Titorenko and Evers, unpublished data). By analogy to the mechanisms of mitochondrial protein import, it can readily be envisaged that saturation of the available peroxisomal assembly factors results in the partial inhibition of protein translocation into the organelle. Because of this secondary effect of FAD depletion, precursors of peroxisomal matrix proteins (e.g., AO, catalase, and dihydroxyacetone synthase) accumulate in the cytosol.

As discussed before, the alternative explanation, namely that the import defect results from a general energy and/or FAD depletion in Rf-limited cells, is highly unlikely.

Thus, the mutant *rif1* to our opinion indeed is ideally suited for a detailed analysis of the sequence of events that occurred after a shift of mutant cells from glucose to methanol-containing media and to discriminate between the primary effect of FAD limitation (inhibition of AO oligomerization) and the secondary effects, namely impairment of peroxisomal protein import and effects on AO synthesis and peroxisome proliferation. As shown before, peroxisomes in *H. polymorpha* are only temporarily competent to import matrix proteins. Under in vivo conditions only small organelles were able to incorporate newly synthesized proteins but lost this capacity upon their maturation (Veenhuis *et al.*, 1989; Waterham *et al.*, 1992b). We therefore speculate that the progressive accumulation of cytosolic precursors during Rf limitation could act as the signal that switches on compensatory regulatory mechanisms as for instance an increase of the general import capacity by the enhanced proliferation of many small import-competent peroxisomes; concomitantly, it may influence the synthesis of peroxisomal matrix proteins. Studies to further elucidate these mechanisms are in progress in our laboratory.

ACKNOWLEDGMENTS

We thank Ineke Keizer and Jan Zagers for assistance in different parts of this study.

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